

The Hsp72 and Hsp90a mRNA responses to hot downhill running are reduced following a prior bout of hot downhill running, and occur concurrently within leukocytes and the vastus lateralis

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Submitted to Journal:
Frontiers in Physiology

Specialty Section:
Exercise Physiology

Article type:
Original Research Article

Manuscript ID:
270198

Received on:
30 Mar 2017

Revised on:
31 May 2017

Frontiers website link:
www.frontiersin.org

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement

J.A.T., P.C.C., L.T., and M.P.L. conception and design of research; J.A.T., J.H.B. D.C.H., A.J.M. O.P., C.K., F.R., and S.A-A. performed experiments; J.A.T., B.C.R.C. O.R.G. P.C.C., A.W.M., L.T., and M.P.L. analyzed data; J.A.T., B.C.R.C. O.R.G. P.C.C., L.T., and M.P.L. interpreted results of experiments; J.A.T. and O.R.G. prepared figures; J.A.T. drafted manuscript; J.A.T., B.C.R.C. O.R.G. J.H.B. D.C.H., A.J.M. O.P., C.K., F.R., S.A-A., P.C.C., A.J.M., A.W.M., L.T., and M.P.L. edited and revised manuscript; J.A.T., B.C.R.C. O.R.G. J.H.B. D.C.H., A.J.M. O.P., C.K., F.R., S.A-A., P.C.C., A.J.M., A.W.M., L.T., and M.P.L. approved the final version of manuscript.

Keywords

Downhill running, heat shock response, Heat stress, heat tolerance, preconditioning, Cross tolerance, thermotolerance.

Abstract

Word count: 338

The leukocyte heat shock response (HSR) is used to determine individual's thermotolerance. The HSR and thermotolerance are enhanced following interventions such as preconditioning and/or acclimation/acclimatisation. However, it is unclear whether the leukocyte HSR is an appropriate surrogate for the HSR in other tissues implicated within the pathophysiology of exertional heat illnesses (eg skeletal muscle), and whether an acute preconditioning strategy (eg downhill running) can improve subsequent thermotolerance. Physically active, non-heat acclimated participants were split into two groups to investigate the benefits of hot downhill running as preconditioning strategy. A hot preconditioning group (HPG; $n = 6$) completed two trials (HPGHOTDOWN1 and HPGHOTDOWN2) of 30 min running at lactate threshold (LT) on -10 % gradient in 30°C and 50 % relative humidity (RH) separated by 7 d. A temperate preconditioning group (TPG; $n = 5$) completed 30 min running at LT on a -1 % gradient in 20°C and 50 % (TPGTEMPFLAT) and 7 d later completed 30 min running at LT on -10 % gradient in 30°C and 50 % RH (TPGHOTDOWN). Venous blood samples and muscle biopsies (vastus lateralis; VL) were obtained before, immediately after, 3, 24 and 48 hr after each trial. Leukocyte and VL Hsp72, Hsp90 α and Grp78 mRNA relative expression was determined via RT-QPCR. Attenuated leukocyte and VL Hsp72 (2.8 to 1.8 fold and 5.9 to 2.4 fold; $p < 0.05$) and Hsp90 α mRNA (2.9 to 2.4 fold and 5.2 to 2.4 fold; $p < 0.05$) responses accompanied reductions ($p < 0.05$) in physiological strain [exercising rectal temperature (-0.3°C) and perceived muscle soreness (~14%)] during HPGHOTDOWN2 compared to HPGHOTDOWN1 (i.e. a preconditioning effect). Both VL and leukocyte Hsp72 and Hsp90 α mRNA increased ($p < 0.05$) simultaneously following downhill runs and demonstrated a strong relationship ($p < 0.01$) of similar magnitudes with one another. Hot downhill running is an effective preconditioning strategy which ameliorates physiological strain, soreness and Hsp72 and Hsp90 α mRNA responses to a subsequent bout. Leukocyte and VL analyses are appropriate tissues to infer the extent to which the HSR has been augmented.

Funding statement

No external funding was received in the preparation of this article.

Ethics statements

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The protocol was approved and conducted in accordance with the University of Bedfordshire's Sport Science and Physical Activity Departmental Human Ethics Committee and all participants signed informed consent in accordance with the ethical standards outlined in the 1964 Declaration of Helsinki.

In review

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Running title: Concurrent leukocyte and vastus lateralis heat shock response

Keywords: Downhill running, heat shock response, heat stress, heat tolerance, preconditioning, cross tolerance, thermotolerance.

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Word count: 7457

Abstract

The leukocyte heat shock response (HSR) is used to determine individual's thermotolerance. The HSR and thermotolerance are enhanced following interventions such as preconditioning and/or acclimation/acclimatisation. However, it is unclear whether the leukocyte HSR is an appropriate surrogate for the HSR in other tissues implicated within the pathophysiology of exertional heat illnesses (eg skeletal muscle), and whether an acute preconditioning strategy (eg downhill running) can improve subsequent thermotolerance. Physically active, non-heat acclimated participants were split into two groups to investigate the benefits of hot downhill running as preconditioning strategy. A hot preconditioning group (HPC; $n = 6$) completed two trials (HPC1_{HOTDOWN} and HPC2_{HOTDOWN}) of 30 min running at lactate threshold (LT) on -10 % gradient in 30°C and 50 % relative humidity (RH) separated by 7 d. A temperate preconditioning group (TPC; $n = 5$) completed 30 min running at LT on a -1 % gradient in 20°C and 50 % (TPC1_{TEMPFLAT}) and 7 d later completed 30 min running at LT on -10 % gradient in 30°C and 50 % RH (TPC2_{HOTDOWN}). Venous blood samples and muscle biopsies (vastus lateralis; VL) were obtained before, immediately after, 3, 24 and 48 hr after each trial. Leukocyte and VL Hsp72, Hsp90 α and Grp78 mRNA relative expression was determined via RT-QPCR. Attenuated leukocyte and VL Hsp72 (2.8 to 1.8 fold and 5.9 to 2.4 fold; $p < 0.05$) and Hsp90 α mRNA (2.9 to 2.4 fold and 5.2 to 2.4 fold; $p < 0.05$) responses accompanied reductions ($p < 0.05$) in physiological strain [exercising rectal temperature (-0.3°C) and perceived muscle soreness (~-14%)] during HPC2_{HOTDOWN} compared to HPC1_{HOTDOWN} (i.e. a preconditioning effect). Both VL and leukocyte Hsp72 and Hsp90 α mRNA increased ($p < 0.05$) simultaneously following downhill runs and demonstrated a strong relationship ($p < 0.01$) of similar magnitudes with one another. Hot downhill running is an effective preconditioning strategy which ameliorates physiological strain, soreness and Hsp72 and Hsp90 α mRNA responses to a subsequent bout. Leukocyte and VL analyses are appropriate tissues to infer the extent to which the HSR has been augmented.

60 List of abbreviations

- 61 C_T - cycling threshold
62 DOMS – Delayed onset muscle soreness
63 Grp78 - Glucose regulated protein 78
64 HOT – Hot testing conditions
65 HPC – Hot preconditioning group
66 Hsp – Heat shock protein (number indicates molecular weight)
67 HSF-1 - Heat Shock factor-1
68 HSR – Heat shock response
69 LT – Lactate threshold
70 mRNA – Messenger RNA
71 PBS – Phosphate-buffered saline
72 QT – Quadriceps tenderness
73 RH – Relative humidity
74 RNA - Ribonucleic acid
75 RPE – Rating of perceived exertion
76 RT-QPCR - Reverse transcription quantitative polymerase chain reaction
77 TEMP – Temperate testing conditions
78 TPC – Temperate preconditioning group
79 TS – Thermal sensation
80 UOsm – Urine Osmolality
81 VL – Vastus lateralis
82 $\dot{V}O_2$ - Oxygen uptake
83 $\dot{V}O_{2max}$ – Maximal oxygen uptake

Introduction

Preconditioning of an individual using environmental stressors, with the intent of ameliorating physiological and cellular stress in extreme conditions has applications for athletic, military and occupational populations (Lee et al., 2014; Taylor et al., 2012). One pathway for preconditioning these populations is the initiation of the heat shock response (HSR) which is characterised by induction of heat shock proteins (Hung et al., 2005; Madden et al., 2008; Taylor et al., 2012). The leukocyte HSR, principally heat shock protein 72 (HSP72; protein and mRNA) is used to indicate the extent of cellular heat acclimation (Amorim et al., 2015), and identify individuals at risk of exertional heat illnesses within athletic, military and occupational settings (Marshall et al., 2007; Moran et al., 2006; Ruell et al., 2007). This is primarily due to the role of Hsp72 mRNA and HSP72 as markers of the cellular stress response and thermotolerance [attenuated cellular stress response suggests a greater likelihood of cellular survival (Kampinga et al., 1995; Theodorakis et al., 1999)] in response to isolated, combined, and cross-environmental stressors (Gibson et al., 2017). Ideally the assessment of thermotolerance would take place in skeletal muscle due to its important role in locomotion and exertional heat illness pathophysiology (Sawka et al., 2011). Unfortunately, obtaining multiple muscle biopsies prior to relocation to a hot environment is not always viable for ethical, performance, cost, comfort and medical reasons (MacInnis et al., 2017). **Leukocytes are a desirable tissue site for determining thermotolerance given the relative ease by which they can be collected, and because leukocytes, as circulating cells, are exposed to both systemic signals and to signals of the perfused tissues (Sonna et al., 2007).** As such Hsp72 mRNA from leukocytes has been utilised as a surrogate to skeletal muscle samples with inferences made from changes in circulating intracellular sites across many exercise, heat, and nutritional experiments whereby the cellular stress response and thermotolerance are augmented (Connolly et al., 2004; Fehrenbach et al., 2000a, 2000b, 2001, Gibson et al., 2015a, 2015c; Marshall et al., 2007; Mee et al., 2016; Niess et al., 2002; Selkirk et al., 2009; Tuttle et al., 2015). Consequently, determining whether the HSR occurs concurrently within both tissues (leukocytes and the vastus lateralis; VL) following an acute stressor (initial experimental trial), and whether this response is attenuated in both tissues following a second trial (i.e. following preconditioning), requires elucidation to assess the viability of the leukocyte HSR to represent the skeletal muscle HSR.

The Hsp72 mRNA response is particularly pertinent during this acute stress response because HSP72 protein concentrations (due to translational inhibition) may not necessarily directly represent the magnitude of the cellular stress response, particularly during the early stages of adaptation to stress (Paulsen et al., 2007) and within heat intolerant individuals (Moran et al., 2006). The differential kinetics of the Hsp72 response in the VL [typically delayed, peak between 24 h and 7d; (Morton et al., 2006; Tupling et al., 2007)] compared to leukocyte subsets [0 - 24 h (Fehrenbach et al., 2000a; Oehler et al., 2001)] suggests the leukocyte Hsp72 mRNA specific response which peaks within 0 - 3 h (Fehrenbach and Northoff, 2001; Neubauer et al., 2014), is more practical (shorter sampling time course required) for assessing the cellular stress response in the VL for comparative purposes. In addition to Hsp72 mRNA, Hsp90 α mRNA is of interest due to its important role within restoration of proteostasis (Kourtis and Tavernarakis, 2011; van Oosten-Hawle et al., 2013), regulation of the transmission of signalling cascades (Taipale et al., 2010), recovery of global protein synthesis (Duncan, 2005) and regulation of cellular repair (Erleijman et al., 2014). Additionally it is unknown if the physiological signals e.g. increases in systemic temperature (Gibson et al., 2016), which elicit increases in leukocyte Hsp72 and Hsp90 α mRNA transcription to damaging (Tuttle et al., 2015), and non-damaging exercise-heat stress (Gibson et al., 2015c), are as relevant in skeletal muscle. The current study also sought to investigate the gene transcript response of another HSP, glucose regulated protein 78 mRNA (Grp78 mRNA) given its ability to indicate when the unfolded protein response ends (Ron and Walter, 2007). Importantly Grp78 mRNA may also act as a biomarker of thermotolerance within heat intolerant individuals where Heat Shock factor-1 (HSF-1) signalling and Hsp72 and Hsp90 α mRNA transcription are attenuated (McMillan et al., 1998). However, it is currently unclear if previous *in vitro* observations demonstrating the role of Hsp72 and Hsp90 α mRNA in the cellular stress response (Heldens et al., 2011) occur within human leukocytes and skeletal muscle (VL) *in vivo* (i.e. following exercise, and exercise and heat related stressors).

Experimental aims were to determine whether a prior bout of hot downhill running [eliciting large changes in exercising rectal temperature (T_{re}) and delayed onset muscle soreness (DOMS)], when compared to a temperate flat run, could provide a preconditioning effect relative to attenuation of the VL Hsp responses (Hsp72, Hsp90 α and Grp78 mRNA) during a subsequent trial of hot downhill running 7 d later. The second experimental aim was to determine whether this response occurred concurrently within leukocytes and the VL. It was hypothesised that a prior bout of hot downhill running would attenuate both the VL and

leukocyte Hsp72 and Hsp90 mRNA responses during a second trial, and that a significant relationship between the VL and leukocyte Hsp72 and Hsp90 mRNA responses following the first trial would exist.

Method

Ethical approval

The protocol was approved by the University of Bedfordshire's Sport Science and Physical Activity Departmental Human Ethics Committee and all participants signed informed consent in accordance with the ethical standards outlined in the 1964 Declaration of Helsinki.

Participants

Demographic variables were recorded for 11 male Caucasian participants (see table 1) who were non-smokers and were not heat acclimated [experimental trials completed between January and March, within the UK; average temperatures 1.5°C – 8.1°C]. Body mass (kg) and height (cm) were measured with a single set of mechanical scales (Weylux Marsden 424 London, UK) and a stadiometer (Harpender HAR- 98.602, Crymych, UK) respectively. Body composition was measured using air displacement plethysmology (Bod Pod 2000A, Cranlea, UK). The lactate threshold (LT) and maximum oxygen uptake ($\dot{V}O_{2\max}$) were determined using a graded treadmill test (Winter et al., 2007). This test consisted of 6 – 8 incremental 3 min stages at a 1 % gradient. Participants started running at 8 - 9 km.h⁻¹ and running velocity was increased by 1 km.h⁻¹ per stage until exhaustion. Fingertip capillary blood samples (40 µL) were taken at rest and the end of each 3 min stage to determine blood lactate concentrations (B[La]). Blood lactate concentrations were plotted against running velocity to determine LT which was defined as the first sustained B[La] increase above baseline. Pulmonary gas exchange was measured breath by breath using an online gas analysis system (Cortex Metalyser 3B, Biophysik, Leipzig, Germany) to determine changes in oxygen uptake ($\dot{V}O_2$) with the highest $\dot{V}O_2$ attained over a 30 s period accepted as $\dot{V}O_{2\max}$.

Sample size calculations of Hsp72 mRNA were determined via G.Power 3.1, (Universität Dusseldorf, Germany) (Faul et al., 2009) using data from a previous paper (Mestre-Alfaro et al., 2012). For a two tailed test with an alpha of 0.05 and power of 0.8, 6 participants were required to find an Hsp72 mRNA increase of 3.8 fold significant. This sample size is \geq others

in the field (Febbraio and Koukoulas, 2000; Fehrenbach et al., 2003; Fehrenbach and Northoff, 2001; Liu et al., 2004; Mee et al., 2016; Puntschart et al., 1996).

Experimental design

Participants were split into two experimental groups (see figure 1). The temperate (TPC; 5 participants) and HOT (HPC; 6 participants) preconditioning groups (conditions) both featured two exercise trials separated by 7 d:

TPC Exercise trial 1): Temperate flat (TPC1_{TEMPFLAT}) which involved 30 min running at the LT on a 1 % gradient in 20°C, 50 % RH. *TPC Exercise trial 2):* 7 d post TPC1_{TEMPFLAT}, hot downhill (TPC2_{HOTDOWN}) which involved 30 min downhill running at the LT on a -10 % gradient in 30°C, 50 % RH.

HPC Exercise trial 1): Hot downhill (HPC1_{HOTDOWN}) which involved 30 min downhill running at the LT on a -10 % gradient in 30°C, 50 % RH. *HPC Exercise trial 2):* 7 d post HPC1_{HOTDOWN}, hot downhill 2 (HPC2_{HOTDOWN}) which involved 30 min downhill running at the LT on a -10 % gradient in 30°C, 50 % RH.

Previous work from our research group has demonstrated that the leukocyte Hsp72 and Hsp90α mRNA responses are larger following exercise in hot compared to temperate environments (Gibson et al., 2015c, 2016), and following downhill compared to flat running (Tuttle et al., 2015). It is known that downhill running is an effective whole body preconditioning strategy (Isanejad et al., 2015; Shima et al., 2008; Touchberry et al., 2012), consequently, an acute preconditioning trial featuring both stressors (hot environmental conditions and downhill running; hot downhill running) was selected in the current experimental design to maximise stimuli to initiate the HSR and subsequent cellular preconditioning. This was compared to a temperate flat trial (flat running in a temperate environment) where no change in leukocyte Hsp72 and Hsp90α mRNA has been previously observed (Tuttle et al., 2015) and thus no preconditioning effect was hypothesised to occur. A 7 d period between trials was selected to ensure any spontaneous preconditioning effect from exercise stress on core temperature (Barnett and Maughan, 1993) and leukocyte HSP72 (Fehrenbach et al., 2001; Lee et al., 2014), had returned to baseline following TPC1_{TEMPFLAT}.

All experimental trials were completed at the running velocity which elicited the LT to minimise differences in metabolic strain between experimental trials (Baldwin et al., 2000).

However, environmental temperature mediated differences still remained as relative exercise intensity is higher at the same velocity during exercise in hot environments (Lorenzo et al., 2011). All experimental trials were completed at the same time of day to minimise the influence of diurnal and circadian variations on exercise performance (Drust et al., 2005). Confounding variables were controlled for via abstinence prior to testing and throughout the testing period (see brackets for duration). These confounding variables were caffeine and alcohol (72 hrs), non-steroidal anti-inflammatory medications [48 hrs (Nielsen and Webster, 1987; Van Wijck et al., 2012)], dietary supplementation (vitamins, ergogenic aids; 30 d), exercise [7 d (Morton et al., 2006)], thermal stressors [3 months (Gibson et al., 2014)] and hypoxic and hyperbaric stressors [3 months (Taylor et al., 2010a, 2011, 2012)]. A questionnaire was administered prior to each experimental trial to determine adherence to the aforementioned experimental control measures with apparent adherence 100 % in all participants.

Participants were instructed to drink 500 mL of water 2 hrs before each experimental trial as per the ACSM position stand (Sawka et al., 2007). Hydration status was assessed via urine osmolality (UOsm) using a handheld digital refractometer (Osmocheck, Vitech Scientific Ltd, Horsham, UK) before any pre exercise measures were obtained and immediately after exercise. All participants were euhydrated [UOsm was < 600 mOsmols.kg.H₂O (Hillman et al., 2011, 2013)] prior to all experimental conditions and remained euhydrated during each experimental trial despite UOsm increasing (Time; $F = 63.7$, $p < 0.001$) immediately post exercise compared to basal.

Place figure 1 near here please

Molecular physiology measures

Blood sampling and leukocyte isolation

Venous blood was obtained from the antecubital vein into a 6 mL EDTA tube immediately before (basal), immediately post, 3 hrs post, 24 hrs post and 48 hrs post exercise. Using an adaptation of a previously validated method (Taylor et al., 2010b), 500 μ L of venous blood was pipetted into 10 mL of 1 in 10 red blood cell lysis solution (10X Red Blood Cell Lysis Solution, Miltenyi Biotech, UK). Samples were incubated for 15 min at room temperature and then isolated via centrifugation at 400G for 5 min and washed twice in 2 mL phosphate-

buffered saline (PBS) at 400 G for 5 min. The pellet was suspended in 1 mL of PBS, pipetted into a 1.5 mL RNase free microtube and then centrifuged at 17 000 G for 5 min at 4°C. The remaining supernatant was aspirated prior to the pellet being completely re-suspended in 200 µL of TRIzol reagent (Sigma Aldrich, Dorset, UK) and stored at -80°C for subsequent RNA extraction.

Muscle biopsies

All biopsies were taken by medically qualified Orthopaedic Surgeons, with full UK General Medical Council registration. Muscle Biopsies were obtained using a previously validated and HSP specific *in vivo* technique (Morton et al., 2006, 2007, 2008, 2009) applied to the lateral portion of the vastus lateralis. Biopsies were taken 3 cm apart in a proximal to distal fashion, under local anaesthetic (2% lidocaine hydrochloride). The fascia of the muscle was specifically avoided (Trappe et al., 2013). Disposable manually primed biopsy needle guns were utilised (12 x 16, Disposable Monopty Core Biopsy Instrument, Bard Biopsy Systems, USA). Samples collected (20-30 mg) were immediately frozen in liquid nitrogen (-196°C) and stored at -80°C for later analysis. Serial biopsies were separated by 3 cm to ensure muscle damage from previous incisions did not influence the Hsp72, Hsp90α and Grp78 mRNA responses (Khassaf et al., 2001).

Biopsy samples were later ground under liquid nitrogen to remove surrounding tissue (i.e. adipose, and connective tissue) prior to homogenisation with a sonicator (T10 Basic, IKA, Thermo Fisher Scientific, Loughborough, UK) on ice in 1 mL TRIzol reagent followed by a 10 min incubation period on ice, in preparation for RNA extraction.

RNA extraction

The TRIzol method was used to extract RNA from the biopsy samples and the leukocytes in accordance with manufacturer instructions (Invitrogen, Life Technologies, Carlsbad, USA). Quantity was determined at an optical density of 260 nm while quality was determined via the 260/ 280 and 260/ 230 ratios using a nanodrop spectrophotometer (Nanodrop 2000c, Thermo Scientific). Only samples with a 260:280 ratio of between 1.9 and 2.15 were carried forward for reverse transcription and PCR amplification detailed below.

One step reverse transcription quantitative polymerase chain reaction (RT-QPCR)

Primers (see table 2) were designed using primer design software (Primer Quest and Oligoanalyzer - Integrated DNA technologies). During primer design sequence homology searches were performed against the Genbank database to ensure the primers matched the gene of interest. Primers were designed to span exon-intron boundaries and avoided three or more GC bases within the last 5 bases at the 3' end of primer to avoid non specific binding. Further searches were performed to ensure primers did not contain secondary structures and inter or intra molecular interactions (hairpins, self-dimer and cross dimers), which can inhibit product amplification. Hsp72, Hsp90 α and Grp78 relative mRNA expression was then quantified using RT-QPCR. 20 μ L reactions containing 10 μ L SYBR-Green RT-PCR Mastermix (Quantifast SYBRgreen Kit, Qiagen, Manchester, UK), 0.15 μ L forward primer, 0.15 μ L reverse primer, 0.2 μ L reverse transcription mix (Quantifast RT Mix, Qiagen) and 9.5 μ L sample (70 ng RNA/ μ L) were prepared using the Qiagility automated pipetting system (Qiagen). Each reaction was amplified in a thermal cycler (Rotorgene Q, Qiagen) and involved reverse transcription lasting 10 min at 50°C and a transcriptase inactivation and initial denaturation phase lasting 5 min at 95°C. The PCR reaction then followed with a denaturation step lasting 10 s at 95°C and a primer annealing and extension stage lasting 30 s at 60°C repeated for 40 cycles. Fluorescence was measured following each cycle as a result of the incorporation of SYBR green dye into the amplified PCR product. Melt curves (50 to 95°C; Ramp protocol 5 s stages) were analysed for each reaction to ensure only the single gene of interest was amplified.

Place table 2 near here please

The relative quantification of mRNA expression for each sample (Hsp72, Hsp90 α and Grp78) was assessed by determining the ratio between the cycling threshold (C_T) value of the target mRNA and the C_T values for β 2-Microglobulin (β 2-M) mRNA. Fold change in relative mRNA expression was calculated using the $2^{-\Delta\Delta C_T}$ method (Schmittgen and Livak, 2008). β 2-Microglobulin was used as a housekeeping gene as it was stable between experimental trials and across time in both the VL and leukocytes, as previously observed following exercise (Mahoney et al., 2004, 2008; Tuttle et al., 2015). The coefficient of variation for β 2-M mRNA, Hsp72 mRNA, Hsp90 α mRNA and Grp78 mRNA were 0.55 %, 0.34 %, and 0.28 % respectively.

Statistical analysis

Central tendency and dispersion are reported as the mean and standard deviation for normally distributed data and as the median and interquartile range for non-normally distributed data. Inferential statistical analyses were completed using linear mixed models for repeated measures (IBM SPSS Statistics 19, Chicago, IL) with comparisons made for main effects, two way interactions (experimental trial x time) and three way interactions (group x experimental trial x time). The best fitting covariance structure was selected by minimising the Hurvich and Tsai's criterion (Field, 2013). Changes in Hsp72, Hsp90 α and Grp78 mRNA are presented as fold change from basal in accordance with previous literature (Gibson et al., 2015c; Tuttle et al., 2015). Where significant F ratios for main and interaction effects occurred, post hoc pairwise comparisons were made with Bonferroni adjusted p values. Pearson's product correlation was performed between leukocyte and vastus lateralis Hsp72 mRNA and Hsp90 α mRNA before, immediately after and 3 hrs after TPC1_{TEMPFLAT} and HPC1_{HOTDOWN}. Pearson's product correlations were also performed between physiological variables T_{re} and HR, and leukocyte and VL Hsp72 mRNA and Hsp90 α mRNA immediately and 3 hrs after the corresponding TPC1_{TEMPFLAT} and HPC1_{HOTDOWN}. The mRNA responses to TPC2_{HOTDOWN} and HPC2_{HOTDOWN} were not included in the correlational analyses given the likelihood of the prior trials to be a confounding factor due to the hypothesised preconditioning effect i.e. increase gene transcription and therefore signal post translational events to increase basal HSP (Tuttle et al., 2015). Statistical significance was accepted at $p < 0.05$ (two tailed).

Results

Thermoregulatory response

Exercising T_{re} (figure 2) increased as main effect between 5 - 30 min ($p < 0.001$) compared to basal. Average exercising T_{re} was higher during the hot downhill running trials (HPC1_{HOTDOWN}; 38.3°C; $F = 14.3$, $p = 0.002$, and TPC2_{HOTDOWN} (37.9°C; $F = 6.1$, $p = 0.017$) compared to the temperate flat trial (TPC1_{TEMPFLAT}; 37.7°C). Exercising T_{re} was greater during the hot downhill trials (TPC2_{HOTDOWN}; 20 - 30 min, $p < 0.05$, HPC1_{HOTDOWN}; 5 - 30 min, $p < 0.05$) compared to the temperate flat trial (TPC1_{TEMPFLAT}). Exercising T_{re} was also 0.3°C higher ($39.3 \pm 0.3^\circ\text{C}$ compared to $39.0 \pm 0.4^\circ\text{C}$) at 30 min during HPC1_{HOTDOWN} compared to HPC2_{HOTDOWN} ($F = 6.1$, $p = 0.017$).

*** Insert figure 2 near here please***

Heart rate (figure 3) was increased compared to basal between 5 - 30 min ($p < 0.001$). Average HR was higher during TPC2_{HOTDOWN} (162 beats.min⁻¹) compared to TPC1_{TEMPFLAT} (147 beats.min⁻¹; $F = 22.3$, $p = 0.001$). No difference in average HR was observed between HPC1_{HOTDOWN} (161 beats.min⁻¹) and TPC1_{TEMPFLAT} ($F = 3.3$, $P = 0.096$) or HPC2_{HOTDOWN} (157 beats.min⁻¹; $F = 2.8$, $p = 0.128$). Heart rate was higher during the hot downhill trials (TPC2_{HOTDOWN}; 5 - 30 min, $p < 0.05$ and HPC1_{HOTDOWN}; 20 - 30 min, $p < 0.05$) compared to the temperate flat trial (TPC1_{TEMPFLAT}). A trend for HR to be reduced during HPC2_{HOTDOWN} compared to HPC1_{HOTDOWN} between 20 – 30 min (8 beats.min⁻¹; $\sim F = 3.8$, $p = \sim 0.069$) was observed.

*** Insert figure 3 near here please***

Perceived muscle soreness (indicated by the VAS; figure 4) was increased over time as a main effect immediately post to 48 hrs post exercise compared to basal ($p < 0.001$). Perceived muscle soreness also increased from basal between immediately post to 48 hrs post exercise following TPC2_{HOTDOWN} and HPC1_{HOTDOWN} ($p < 0.001$) and between immediately post – 3 hrs post HPC2_{HOTDOWN} ($p < 0.05$). Perceived muscle soreness was greater following the hot downhill running trials (TPC2_{HOTDOWN} and HPC1_{HOTDOWN}) compared to the temperate flat running trial (TPC1_{TEMPFLAT}) immediately post, ($F = 7.2$, $p = 0.011$ and $F = 11.8$, $p = 0.002$), 3 hrs post ($F = 6.1$, $p = 0.019$ and $F = 9.1$, $p = 0.005$), 24 hrs post ($F = 12.2$, $p = 0.001$ and $F = 25.0$, $p < 0.001$) and 48 hrs post exercise ($F = 14.3$, $p = 0.001$ and $F = 30.4$, $p < 0.001$) respectively. Perceived muscle soreness was attenuated 24 hrs and 48 hrs after the second hot downhill running trial (HPC2_{HOTDOWN}) compared to the first hot downhill trial (HPC1_{HOTDOWN}; $F = 12.6$, $p = 0.001$ and $F = 11.3$, $p = 0.002$ respectively) in the HPC.

Quadriceps tenderness (QT; table 3) was increased as a main effect immediately post to 48 hrs post exercise ($p < 0.05$) compared to basal. No difference in QT was observed between experimental trials ($P > 0.05$).

Insert figure 4 near here please

Metabolic and perceptual responses

Compared to basal, blood lactate concentrations (table 3) increased following the hot downhill running trials (TPC2_{HOTDOWN}; $F = 11.0$, $p = 0.006$, HPC1_{HOTDOWN}; $F = 13.3$, $p = 0.003$ and HPC2_{HOTDOWN}; $F = 5.7$, $p = 0.035$), but not the temperate flat trial (TPC1_{TEMPFLAT}; $F = 0.0$, $p = 0.874$). Oxygen uptake ($\dot{V}O_2$) increased ($F = 236.0$, $p < 0.001$) over time as a main effect but there was no difference between experimental trials ($P < 0.05$). Participants exercised at an average % $\dot{V}O_{2max}$ of $70.2 \pm 6.0\%$ during the TPC1_{TEMPFLAT} trial, $70.8 \pm 6.9\%$ during the TPC2_{HOTDOWN} trial, $66.2 \pm 6.0\%$ during the HPC1_{HOTDOWN} trial and $65.8 \pm 8.4\%$ during the HPC2_{HOTDOWN} trial.

Both the rate of perceived exertion (RPE; table 3) and thermal sensation (TS; table 3) were greater during the hot downhill running trials (TPC2_{HOTDOWN} and HPC1_{HOTDOWN}; $p < 0.05$) compared to the temperate flat trial (TPC1_{TEMPFLAT}). No difference in RPE or TS was observed between the HPC1_{HOTDOWN} and HPC2_{HOTDOWN} trials ($p > 0.05$).

*** Insert table 3 near here please***

Cellular stress (Hsp mRNA) response

The responses of Hsp72, Hsp90 α and Grp78 mRNA were assessed to determine their suitability as markers of the cellular stress response. Vastus lateralis Hsp72 mRNA (figure 5A) increased as a main effect immediately post ($p < 0.001$) and 3 hrs post exercise ($p = 0.002$) compared to basal. Vastus lateralis Hsp72 mRNA increased immediately post exercise compared to basal in the hot downhill running trials (TPC2_{HOTDOWN} and HPC1_{HOTDOWN}; $p < 0.001$). Vastus lateralis Hsp72 mRNA expression was greater immediately post TPC2_{HOTDOWN} and HPC1_{HOTDOWN} compared to the temperate flat trial (TPC1_{TEMPFLAT}; $F = 24.2$, $p < 0.001$ and $F = 9.2$, $p = 0.004$ respectively) and the second hot downhill trial in the hot preconditioning group (HPC2_{HOTDOWN}; $F = 9.7$, $p = 0.003$ and $F = 5.0$, $p = 0.028$, respectively). Vastus lateralis Hsp72 mRNA was also greater 3 hr after HPC1_{HOTDOWN} compared to TPC1_{TEMPFLAT} ($F = 6.6$, $p = 0.013$).

Leukocyte Hsp72 mRNA expression (figure 5B) increased as a main effect immediately post ($p < 0.001$) and 3 hrs post exercise ($p = 0.004$) compared to basal. Leukocyte Hsp72 mRNA expression was greater following HPC1_{HOTDOWN} compared to TPC1_{TEMPFLAT} ($F = 4.2$, $p = 0.049$) and HPC2_{HOTDOWN} ($F = 10.2$, $p = 0.003$).

Place figure 5 near here please

Vastus lateralis Hsp90 α mRNA (figure 6A) increased compared to basal following the hot downhill running trials TPC2_{HOTDOWN} (immediately post exercise; $p < 0.001$) and HPC1_{HOTDOWN} (immediately post; $p < 0.001$ and 3 hr post; $p = 0.020$). Vastus lateralis Hsp90 α mRNA expression was greater immediately post TPC2_{HOTDOWN} compared to TPC1_{TEMPFLAT} ($F = 8.4$, $p = 0.006$), and HPC2_{HOTDOWN} ($F = 7.4$, $p = 0.010$). Vastus lateralis Hsp90 α mRNA expression was also greater following HPC1_{HOTDOWN} compared to TPC1_{TEMPFLAT} (immediately post; $F = 4.3$, $p = 0.044$ and 3 hrs post; $F = 4.4$, $p = 0.043$) and HPC2_{HOTDOWN} (immediately post; $F = 19.4$, $p < 0.001$).

Leukocyte Hsp90 α mRNA expression increased as a main effect immediately post exercise compared to basal ($p < 0.001$). Leukocyte Hsp90 α mRNA expression also increased following TPC2_{HOTDOWN} (immediately post; $p = 0.024$) and HPC1_{HOTDOWN} (immediately post; $p < 0.001$ and 3 hr post; $p = 0.041$) compared to basal. Leukocyte Hsp90 α mRNA expression was greater immediately after TPC2_{HOTDOWN} compared to TPC1_{TEMPFLAT} ($F = 5.3$, $p = 0.024$). Leukocyte Hsp90 α mRNA expression was also greater immediately after HPC1_{HOTDOWN} compared to TPC1_{TEMPFLAT} ($F = 10.1$, $p = 0.002$) and HPC2_{HOTDOWN} ($F = 4.9$, $p = 0.030$).

Place figure 6 near here please

Vastus lateralis Grp78 mRNA (figure 7A) increased as a main effect immediately post to 48 hrs post exercise compared to basal ($p < 0.002$). Vastus lateralis Grp78 mRNA also increased within the TPC immediately post ($p = 0.003$) and within the HPC at 3 hrs ($p < 0.001$) and 24 hrs post ($p < 0.001$). Vastus lateralis Grp78 mRNA increased compared to basal following the hot downhill running trials, TPC2_{HOTDOWN} (immediately post; $p < 0.001$), HPC1_{HOTDOWN} (3 hr and 24 hr post; $p < 0.010$) and HPC2_{HOTDOWN} (24 hr post; $p = 0.003$), but did not change following the temperate flat trial (TPC1_{TEMPFLAT}; $p > 0.05$).

All main effects and interactions had no effect ($p > 0.05$) on leukocyte Grp78 mRNA (figure 7B).

Place figure 7 near here please

Relationship between mRNA responses

A strong correlation was observed between vastus lateralis Hsp72 and Hsp90 α mRNA expression ($r = 0.863$, $p < 0.001$; Figure 8A), and between leukocyte Hsp72 and Hsp90 α

mRNA expression ($r = 0.844$, $p < 0.001$; Figure 8B). Modest correlations were also observed between leukocyte Hsp72 mRNA and vastus lateralis Hsp72 mRNA ($r = 0.651$, $p < 0.001$; Figure 8C), and between leukocyte Hsp90 α mRNA and vastus lateralis Hsp90 α mRNA ($r = 0.640$, $p < 0.001$; Figure 8D). Relationships between Hsp72 and Hsp90 α mRNA, and Grp78 mRNA were not analysed given the absence of a change in leukocyte Grp78 mRNA (figure 7B).

Place figure 8 near here please

A strong relationship was also observed between the peak T_{re} during TPC1_{TEMPFLAT} and HPC1_{HOTDOWN} and the immediately post measured leukocyte Hsp72 ($r = 0.665$, $p = 0.026$) and Hsp90 α mRNA ($r = 0.708$, $p = 0.015$), and the 3 hrs measured leukocyte ($r = 0.786$, $p = 0.004$) and vastus lateralis ($r = 0.720$, $p = 0.013$) Hsp72 mRNA, and vastus lateralis Hsp90 α mRNA ($r = 0.682$, $p = 0.021$). A strong relationship was also observed between peak heart rate during TPC1_{TEMPFLAT} and HPC1_{HOTDOWN}, and leukocyte ($r = 0.739$, $p = 0.009$) and vastus lateralis ($r = 0.766$, $p = 0.006$) Hsp72 mRNA, and leukocyte ($r = 0.677$, $p = 0.022$) and vastus lateralis Hsp90 α mRNA ($r = 0.746$, $p = 0.008$) at 3hrs post exercise. No significant relationship was observed immediately post TPC1_{TEMPFLAT} or HPC1_{HOTDOWN}.

Discussion

The current study demonstrated that both VL and leukocyte Hsp72 and Hsp90 α mRNA increases following the first trial of downhill running in a hot environment (HPC1_{HOTDOWN}) were attenuated concurrently with reductions in exercising T_{re} and DOMS during the second trial of downhill running in a hot environment (HPC2_{HOTDOWN}; see figure 5 and 6). This suggests that the cellular stress response (Hsp72 and Hsp90 α mRNA) occurred simultaneously within both tissues (Figure 8) and likely contributed to the preconditioning effect. This was not demonstrated in GRP78 mRNA (Figure 7). The absence of change in GRP78 mRNA in leukocytes suggests this is not an appropriate tissue to determine changes in its expression levels. Therefore the leukocyte Hsp72 and Hsp90 α mRNA responses could potentially be a useful surrogate for the VL response. At a physiological level the attenuated T_{re} (Figure 2), HR (Figure 3) and VAS (Figure 4) responses to an equivalent downhill run following HPC demonstrates an acute preconditioning response was attained. This was not discernible in the TPC group whom demonstrated the known responses to downhill running

under heat stress in comparison to level gradient running in temperate conditions i.e. increased T_{re} (Figure 2), HR (Figure 3) and VAS (Figure 4).

Cellular stress response and surrogate Hsp mRNA response

Increased Hsp72 mRNA transcription has frequently been demonstrated following exercise [leukocytes and VL (Mestre-Alfaro et al., 2012; Walsh et al., 2001)], muscle damaging exercise [VL (Vissing et al., 2009)] and exercise heat stress [leukocytes (Mestre-Alfaro et al., 2012)] within humans. However, there is less data available regarding the Hsp72 mRNA response being attenuated during repeated trials of muscle damaging exercise, or exercise heat stress as observed frequently during repeated trials of *in vitro* heat shock (Kiang et al., 1996; Theodorakis et al., 1999). Studies have previously only observed a blunted response following muscle damaging exercise in the VL (Paulsen et al., 2007) and exercise heat stress within leukocytes (Fehrenbach et al., 2001; Marshall et al., 2007). Within these studies reductions in thermal strain [exercising T_{re} (Fehrenbach et al., 2001; Marshall et al., 2007)] and muscle damage (Paulsen et al., 2007) during subsequent experimental trials were suggested to be responsible for the attenuated Hsp72 mRNA response observed. The current study also observed a reduction in thermal strain (T_{re} -0.3°C) equivalent to that of various heat acclimation regimes (Gibson et al., 2015b; Tyler et al., 2016), and an attenuated perceived muscle soreness (24hr post = +12.2%, 48 hr post = -16.5%) response that is indicative of muscle damage (Fridén et al., 1981) from near identical exercise trials [HPC2_{HOTDOWN} compared to HPC1_{HOTDOWN} (see figure 2 and table 3)]. Together these responses indicate that downhill running models may be able to elicit a beneficial preconditioning effect (Dolci et al., 2015; Tuttle et al., 2015). Given that acute non-damaging exercise heat stress does not improve thermal responses to a greater extent than equivalent temperate condition exercise [Figure 2, (Lee et al., 2014)], the cellular responses to the eccentric muscle action of the damaging downhill running is important. The attenuated exercising T_{re} response could be suggestive of a reduction in relative exercise intensity and therefore potentially reduced requirement for ATP production (Febbraio et al., 1996), though no statistical difference in absolute intensity as indicated by $\dot{V}O_2$ was observed (Table 3). Therefore, metabolic strain was likely reduced. Protein denaturation, the key cellular change associated with heat shock factor-1 (HSF-1) activation and Hsp72 and Hsp90 α mRNA transcription, is temperature (Mestre-Alfaro et al., 2012), metabolic strain (Beckmann et al.,

1992) and muscle damage (Michailidis et al., 2013) dependent. This suggests the observed attenuated thermal strain and muscle damage responses could be an important mechanism explaining the attenuated Hsp72 mRNA response in leukocytes ($HPC1_{HOTDOWN} = +207\%$; $HPC2_{HOTDOWN} = +79\%$) and VL ($HPC1_{HOTDOWN} = +353\%$; $HPC2_{HOTDOWN} = +109\%$) observed following the $HPC2_{HOTDOWN}$ trial, compared to $HPC2_{HOTDOWN}$ trial. Although the expression of VL (Neubauer et al., 2014) and leukocyte (Moran et al., 2006) Hsp90 α mRNA have previously been observed to increase following exercise and exercise heat stress respectively, with equality of physiological stimuli i.e. T_{re} maintaining Hsp90 α mRNA transcription (Gibson et al., 2015c), no studies have determined whether Hsp90 α mRNA is attenuated during repeated trials of muscle damaging exercise. Consequently, the attenuated Hsp90 α mRNA response in both leukocytes ($HPC1_{HOTDOWN} = +106\%$, $HPC2_{HOTDOWN} = +45\%$) and skeletal muscle ($HPC1_{HOTDOWN} = +122\%$, $HPC2_{HOTDOWN} = +113\%$) following reductions in physiological strain is a novel observation (see Figure 6). It is a novel finding that the relationship between Hsp72 and Hsp72 mRNA transcription is equivalent in the VL (Figure 8A, $R^2 = 0.74$), as has been previously shown in leukocytes [$R^2 = 0.77$ (Gibson et al., 2016)]. It has also been observed that the Hsp72 and Hsp72 mRNA transcription response is comparable following damaging exercise (Figure 8B, $R^2 = 0.71$), as it has previously in non-damaging exercise models (Gibson et al., 2016). Within leukocytes, it has been observed that Hsp72 mRNA transcription (Gibson et al., 2015a, 2015c; Mee et al., 2016), and Hsp90 α mRNA transcription (Gibson et al., 2015c) returns to baseline 24 h following non-damaging exercise heat stress (Moran et al., 2006). The heat shock factor-1 (HSF-1) transcription pathway likely highlights the mechanism between equality of increases in Hsp72 and Hsp90 α mRNA as demonstrated in this experiment (Figure 8), and others utilising a non-damaging model (Gibson et al., 2016) with the attenuated mRNA response in the $HPC2_{HOTDOWN}$ trial reflecting a reduction in the physiological stimuli as a result of the prior HPC for all participants (Figure 9).

HSP72 protein concentrations (due to translational inhibition) may not necessary directly represent the magnitude of the cellular stress response therefore the mRNA response has been proposed as more appropriate (Amorim et al., 2015; Gibson et al., 2015a; Lee et al., 2015). A reduction in the mRNA response is therefore representative of a gain in protein concentration (Marshall et al., 2007). The VL cellular adaptations associated with the repeated bout effect include a strengthened cytoskeleton [increased desmin concentrations (Feasson et al., 2002)] and elevated small HSP concentrations [$\alpha\beta$ -crystallin and HSP27 (Paulsen et al., 2009)] and

therefore, could be responsible for the attenuated Hsp72 and Hsp90 α mRNA responses observed following HPC2_{HOTDOWN}. Optimisation of transcriptional and translational processes (Touchberry et al., 2012) and elevated concentrations of anti-apoptotic (Horowitz, 2014) and antioxidant (Horowitz and Kodesh, 2010) proteins, which are implicated in enhanced thermotolerance, could also be responsible for the attenuated Hsp72 and Hsp90 α mRNA responses observed following HPC2_{HOTDOWN} within both the VL and leukocytes.

The current study observed for the first time that the leukocyte and VL Hsp72 and Hsp90 α mRNA response occurs concurrently (Figure 8C and 8D). **This novel data supports the notion that leukocytes are a desirable tissue site for determining the cellular stress response due to accessibility for analysis following exposure to both systemic signals and to signals of the perfused tissues (Sonna et al., 2007). Some caution should be raised as this experiment did not quantify the leukocyte infiltration to skeletal muscle, a known component of the intramuscular response which follows damaging exercise (Malm et al., 2004), though the time course and magnitude of this response are controversial (St. Pierre Schneider and Tiidus, 2007). A resolution to this issue within future experiments would be quantification of total mRNA (Sanders et al., 2014).** As previously discussed the reduction in thermal and metabolic strain mediated within both leukocytes and the VL likely attenuated the increases in protein denaturation during HPC2_{HOTDOWN} and thus could explain the attenuated Hsp72 and Hsp90 α mRNA response observed in both tissues. Muscle damage mediated release of ligands [damage associated molecular patterns (DAMPs), circulating cell free DNA and extracellular HSPs (Neubauer et al., 2014)] from skeletal muscle could also explain the concurrent Hsp72 and Hsp90 α mRNA responses via a toll like receptor mediated stress response within leukocytes, as previously observed following muscle damaging exercise (Fernandez-Gonzalo et al., 2012). Although elevations in these ligands may be exercise related (Neubauer et al., 2013), evidence for these ligands actually being released from skeletal muscle following exercise is limited. Consequently, the concurrent Hsp72 and Hsp90 α mRNA responses are probably dependent on increases in thermal strain and metabolic strain within both leukocytes and the VL, and are unlikely to be muscle damage dependent.

Increases in VL Grp78 mRNA were observed following both HPC1_{HOTDOWN} and HPC2_{HOTDOWN} despite the observed reductions in exercising T_{re} and DOMS, which are associated with reduced protein denaturation, the key cellular change regulating Grp78

mRNA transcription. Activation of the unfolded protein response also occurs when the endoplasmic reticulum protein load increases during cellular remodelling (Ron and Walter, 2007). Therefore, the Grp78 mRNA response may reflect the need to increase ER protein folding capacity to aid cellular adaptation (Ron and Walter, 2007). These observations combined with the absence of Grp78 mRNA increases within leukocytes suggest that Grp78 mRNA cannot be used as a marker of the cellular stress response, or thermotolerance, at least within the current experimental model.

Practical applications and future directions

The results of this experiment highlight that an acute bout of downhill running in a hot environment is an effective preconditioning strategy to attenuate the increase in thermal strain experienced during a subsequent, equivalent exercise in hot conditions. Typically it is proposed that athletes, workers and the military should perform acclimation/acclimatization prior to travelling to unfamiliar, hot conditions (Racinais et al., 2015). An acute bout of downhill running in hot conditions i.e. whole body preconditioning may therefore be there an appropriate method to expediently elicit thermal protection i.e. a reduction in thermal strain prior to exercise in hot conditions. Given recent evidence of cross acclimation between stressors (Gibson et al., 2015c; Lee et al., 2016; White et al., 2016), it is also possible that this whole body preconditioning strategy will induce physiological and cellular adaptations which are beneficial in unfamiliar stressors e.g. hypoxia. These adaptations may become greater with repeated stress, i.e. repeated HPC, thus providing either a greater magnitude of cytoprotection, or a more prolonged post-HPC level of protection, or a combination of both. It is currently unknown how long the preconditioning effect elicited by HPC_{1HOTDOWN} is retained beyond the 7 d duration we have observed. Without evidencing the decay in HSP72 and HSP90 α content this is difficult to estimate, as such this remains an area for future investigation. Measurement of RNA/protein ratios may also aid understanding of the cytoprotective dynamics. The current study suggests that the leukocyte Hsp72 and Hsp90 α mRNA responses could potentially be used as a surrogate measure of the HSR within skeletal muscle, at least within the current experimental model (preconditioning via downhill running in a hot environment). Consequently, the leukocyte Hsp72 and Hsp90 α mRNA responses are potentially a relevant marker of individuals thermotolerance and thus could be useful for allocating appropriate athletic or occupational workloads without the potential reductions in performance and increased infection risk (within the biopsy incision) associated with skeletal muscle biopsies. The current experimental model utilised a combination of exercise heat

stress and downhill running. Consequently, leukocyte Hsp72 mRNA and Hsp90 α mRNA responses could be useful for suggesting thermotolerance within situations where exercise heat stress occur, such as military exercises or during athletic competition. Although the concurrent Hsp72 and Hsp90 α mRNA responses are unlikely to be mechanistically linked exclusively to a muscle damage response, future work should set out to confirm whether this concurrent leukocyte and skeletal muscle response also occurs within a non-damaging exercise heat stress trial.

Summary and Conclusions

Hot downhill running is an effective preconditioning strategy which ameliorates physiological strain, muscle soreness and the cellular stress response (Hsp72 and Hsp90 α mRNA transcription) to a subsequent bout of exercise-heat stress. This preconditioning strategy has applications for athletic, occupational and military populations. The current study suggests that Hsp72 and Hsp90 α mRNA act as markers of the cellular stress response within both the VL and leukocytes. Consequently, the leukocyte Hsp72 mRNA and Hsp90 α mRNA responses appear to be a surrogate measure of the cellular stress response in the VL. Accordingly, venepuncture to obtain circulating leukocytes provides a viable alternative to muscle sampling via biopsies to determine the cellular stress response to exercise-heat stress.

Funding

No external funding was received in the preparation of this article.

Acknowledgements

The authors would like to thank the participants who took part in this study. For M.P.L., this activity was conducted under the auspices of the National Centre for Sport and Exercise Medicine (NCSEM), collaboration between several universities, NHS trusts and sporting and public bodies. The views expressed are those of the author and not necessarily those of NCSEM or the partners involved.

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615 approved the final version of manuscript.

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918 **Tables**

919 **Table 1 Participant demographic characteristics**

	Temperate preconditioning group (TPC; n = 5)	Heat preconditioning group (HPC; n = 6)	Group sig (p < 0.05)
Age (Years)	20.4 \pm 2.8	21.7 \pm 2.3	0.426
Height (cm)	177 \pm 7	180 \pm 10	0.593
Body Weight (kg)	75.2 \pm 18.1	76.1 \pm 12.3	0.931
$\dot{V}O_2$ max (mL.kg.min ⁻¹)	50.8 \pm 6.9	52.8 \pm 5.0	0.587
% Lean mass	88.3 \pm 11.5	86.8 \pm 4.8	0.777
% Body Fat	11.7 \pm 11.5	13.2 \pm 4.8	0.777

920 Values are expressed as mean \pm SD. mL.kg.min⁻¹ (millilitres per kilogram per minute),
921 $\dot{V}O_{2\max}$ (maximum oxygen uptake).

In review

922 Table 2 Primer sequences.

Gene	NCBI Accession #	Primer	Sequence (5' → 3')	Amplicon length
β2- Microglobulin (β2-M)	NM_004048	Forward	CCGTGTGAACCATGTGACT	91
		Reverse	TGCGGCATCTTCAAACCT	
Grp78	NM_005347	Forward	TGGAGGTGGGCAAACAAAG ACA	154
		Reverse	TGCTTGGCGTTGGGCATCAT TA	
Hsp72	NM_005345	Forward	CGCAACGTGCTCATCTTTGA	198
Hsp90α (variant 1 & variant 2)	NM_001017 963 & NM_005348	Forward	TCGCTTGTTCTGGCTGATGT	180
		Reverse	AAACTGCGCTCCTGTCTTCT TGC GTGATGTGTCGTCATCT	

923 3' (3 primer end), 5' (5 primer end), Grp78 (Glucose regulated protein 78), Hsp72 (Heat
924 shock protein 72), Hsp90α (Heat shock protein 90 α).

Table 3 Physiological and perceptual responses

		TPC1 _{TEMPFLAT}	TPC2 _{HOTDOWN}	HPC1 _{HOTDOWN}	HPC2 _{HOTDOWN}		
B[La] (mmol.l ⁻¹)	Basal	1.0 ± 0.3	0.8 ± 0.1	0.6 ± 0.1	0.8 ± 0.2		
	Immediately post	1.0 ± 0.3	1.8 ± 0.5*	1.6 ± 0.8*	1.1 ± 0.6*		
Urine Osmolality (mOsmols.kg H ₂ O)	Basal	250.0 ± 200.0	150.0 ± 40.0	165.0 ± 152.5	170.0 ± 185.0		
	Immediately post	310.0 ± 60.0 ^B	330.0 ± 100.0 ^B	390.0 ± 267.5 ^B	430.0 ± 305.0 ^B		
Quadriceps Tenderness (% decrease in force required to elicit tenderness)	Basal	100 ± 0.0	98.9 ± 10.9	100 ± 0.0	101.4 ± 8.7		
	Immediately Post	87.8 ± 9.3	79.3 ± 16.7	85.3 ± 12.5	87.0 ± 11.3		
	3 hrs post	86.6 ± 15.6	82.3 ± 14.7	88.6 ± 7.7	92.3 ± 8.8		
	24 hrs post	88.9 ± 13.8	79.9 ± 22.5	73.1 ± 9.7	85.3 ± 4.8		
	48 hrs post	93.6 ± 7.9	89.0 ± 27.9	82.8 ± 16.9	99.3 ± 10.5		
$\dot{V}O_2$ (ml.kg.min ⁻¹)	0 min	5 min	10 min	15 min	20 min	25 min	30 min
TPC1 _{TEMPFLAT}	6.4 ± 1.8	34.3 ± 4.2*	35.1 ± 5.6*	36.2 ± 6.4*	36.1 ± 5.6*	35.9 ± 5.0*	36.0 ± 4.0*
TPC2 _{HOTDOWN}	6.0 ± 1.0	34.2 ± 6.2*	35.1 ± 6.5*	36.1 ± 7.8*	36.7 ± 7.8*	36.1 ± 6.8*	36.2 ± 7.2*
HPC1 _{HOTDOWN}	6.3 ± 1.5	32.0 ± 1.8*	32.6 ± 2.1*	34.4 ± 1.7*	35.6 ± 1.4*	36.7 ± 1.8*	37.2 ± 1.7*
HPC2 _{HOTDOWN}	6.5 ± 0.8	32.0 ± 1.3*	33.4 ± 1.6*	33.6 ± 2.5*	35.4 ± 2.4*	36.0 ± 2.7*	36.4 ± 2.8*
RPE (Units)	0 min	5 min	10 min	15 min	20 min	25 min	30 min
TPC1 _{TEMPFLAT}	6.0 ± 0.0	10.0 ± 2.0	10.4 ± 0.8	11.8 ± 1.5	12.6 ± 1.5	13.2 ± 1.6	13.4 ± 1.6
TPC2 _{HOTDOWN}	6.0 ± 0.0	11.4 ± 1.3 ^D	13.4 ± 0.9 ^D	14.4 ± 0.6 ^D	15.4 ± 0.9 ^D	16.0 ± 0.8 ^D	17.3 ± 1.0 ^D
HPC1 _{HOTDOWN}	6.0 ± 0.0	11.5 ± 1.1 ^D	13.2 ± 0.8 ^D	14.2 ± 1.1 ^D	15.3 ± 0.5 ^D	16.5 ± 0.6 ^D	17.5 ± 1.2 ^D
HPC2 _{HOTDOWN}	6.0 ± 0.0	11.8 ± 0.8	12.8 ± 0.8	13.7 ± 1.0	15.1 ± 1.0	16.0 ± 1.3	17.0 ± 1.3
TS (Units)	0 min	5 min	10 min	15 min	20 min	25 min	30 min
TPC1 _{TEMPFLAT}	4.0 ± 0.0	3.6 ± 1.1	4.4 ± 1.0	5.0 ± 0.9	5.4 ± 0.7	5.5 ± 0.6	5.5 ± 0.7
TPC2 _{HOTDOWN}	4.0 ± 0.0	5.1 ± 0.7 ^D	5.6 ± 0.7 ^D	6.0 ± 0.7 ^D	6.6 ± 0.7 ^D	7.0 ± 0.4 ^D	7.3 ± 0.3 ^D
HPC1 _{HOTDOWN}	4.0 ± 0.0	4.7 ± 0.5 ^D	5.3 ± 0.4 ^D	5.8 ± 0.5 ^D	6.3 ± 0.4 ^D	6.8 ± 0.4 ^D	6.9 ± 0.4 ^D
HPC2 _{HOTDOWN}	4.0 ± 0.0	4.8 ± 0.4	5.5 ± 0.7	5.7 ± 0.7	6.3 ± 0.4	6.7 ± 0.4	6.8 ± 0.4

Values are expressed as mean ± SD for quadriceps tenderness and $\dot{V}O_2$. Values are expressed as median ± IQR for B[La], RPE, TS and Urine Osmolality. * (increased compared to basal (p < 0.05), ^A (increased compared to HPC1_{HOTDOWN}), ^B (increased from basal (main effect)), ^C (Increased in HPC compared to TPC), ^D (Increased compared to TPC1_{TEMPFLAT}).

Figure Legends

Figure 1 Schematic representation of the experimental design. $\dot{V}O_{2\max}$ (maximum oxygen uptake).

Figure 2 Rectal temperature (T_{re}) at 0 min to 30 min of exercise. A, T_{re} increased ($p < 0.005$) during TPC2_{HOTDOWN} compared to TPC1_{TEMPFLAT}. B, T_{re} decreased ($p = 0.017$) during HPC2_{HOTDOWN} compared to HPC1_{HOTDOWN}. Mean data presented. Error bars omitted to maintain clarity.

Figure 3 Heart rate (HR) at 0 min to 30 min of exercise. A, increased ($p < 0.010$) during TPC2_{HOTDOWN} compared to TPC1_{TEMPFLAT} at 5 - 30 min. B, increased ($p < 0.050$) during HPC1_{HOTDOWN} increased compared to TPC1_{TEMPFLAT} at 20 - 30 min. Mean data presented. Error bars omitted to maintain clarity.

Figure 4 Perceived muscle soreness measured via the Visual analog scale of pain (VAS) immediately before, immediately post, 3 hrs post, 24 hrs post and 48 hrs post exercise. A, increased ($p < 0.005$) during TPCHEAT_{DOWN} compared to TPC1_{TEMPFLAT}. B, increased ($p < 0.001$) during HPC1_{HOTDOWN} compared to TPC1_{TEMPFLAT}. C, decreased ($p < 0.001$) during HPC2_{HOTDOWN} compared to HPC1_{HOTDOWN}. Median data presented. Error bars omitted to maintain clarity.

Figure 5 Hsp72 mRNA response immediately before, immediately post, 3 hrs post, 24 hrs post and 48 hrs post exercise in the Vastus lateralis (5A) and Leukocytes (5B). * Increased ($p < 0.001$) compared to basal. A increased ($p = 0.020$) in HPC1_{HOTDOWN} compared to TPC1_{TEMPFLAT}. B increased ($p < 0.001$) in TPC2_{HOTDOWN} compared to TPC1_{TEMPFLAT}. C increased ($p = 0.028$) in HPC1_{HOTDOWN} compared to HPC2_{HOTDOWN}. D increased in TPC2_{HOTDOWN} compared to HPC2_{HOTDOWN}. E HPC1_{HOTDOWN} increased ($p = 0.049$) compared to HPC2_{HOTDOWN}. F HPC1_{HOTDOWN} increased ($p = 0.003$) compared to TPC1_{TEMPFLAT}. Data presented as median \pm interquartile range.

Figure 6 Hsp90 α mRNA response immediately before, immediately post, 3 hrs post, 24 hrs post and 48 hrs post exercise in the Vastus lateralis (6A) and Leukocytes (6B). * Increased ($p < 0.02$) compared to basal. A increased ($p = 0.006$) during TPC2_{HOTDOWN} compared to TPC1_{TEMPFLAT}. B increased ($p < 0.001$) during HPC1_{HOTDOWN} compared to HPC2_{HOTDOWN}. C increased ($p < 0.05$) during HPC1_{HOTDOWN} compared to TPC1_{TEMPFLAT}. D increased ($p = 0.01$) during TPC2_{HOTDOWN} compared to HPC2_{HOTDOWN}. E ($p = 0.024$) during TPC2_{HOTDOWN} compared to TPC1_{TEMPFLAT}. F increased ($p = 0.030$) during HPC1_{HOTDOWN} compared to HPC2_{HOTDOWN}. G ($p = 0.002$) during HPC1_{HOTDOWN} compared to TPC1_{TEMPFLAT}. Data presented as median \pm interquartile range.

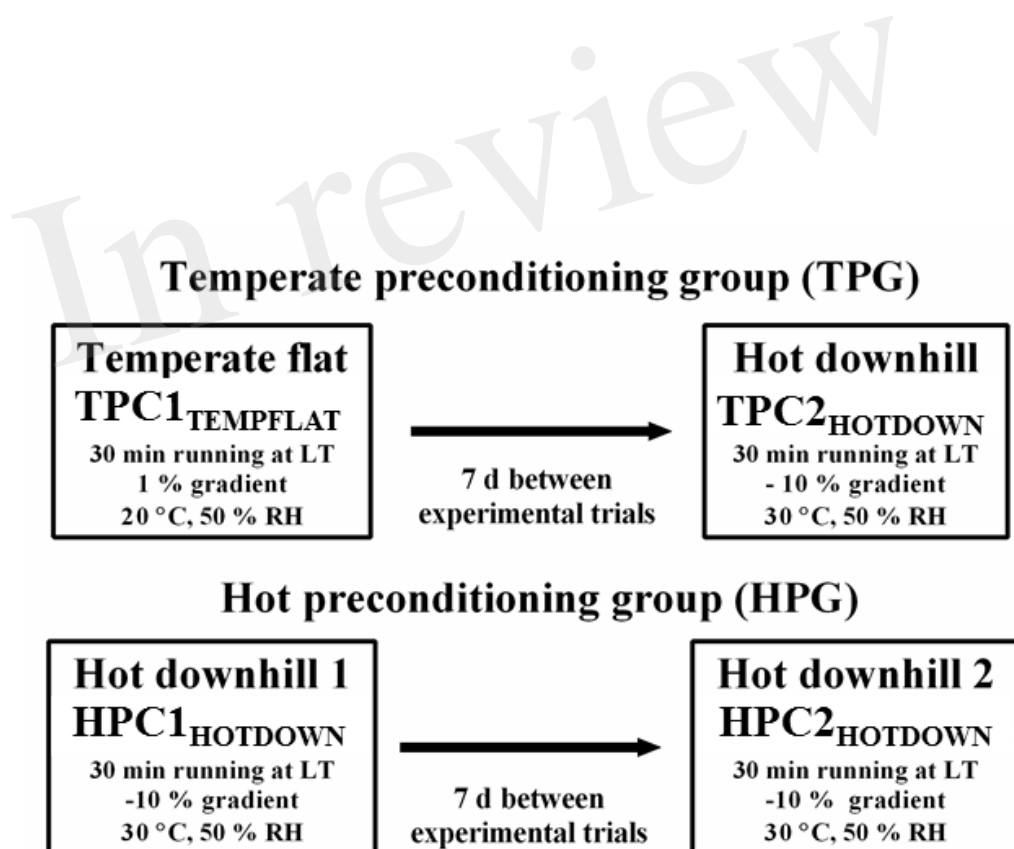
Figure 7 Grp78 mRNA response immediately before, immediately post, 3 hrs post, 24 hrs post and 48 hrs post exercise in the Vastus lateralis (7A) and Leukocytes (7B). * Increased ($p < 0.01$) compared to basal. * Increased ($p < 0.01$) compared to basal. A increased ($p = 0.031$) during HPC1_{HOTDOWN} compared to TPC1_{TEMPFLAT}. B increased ($p < 0.001$) during HPC compared to TPC. C decreased ($p = 0.006$) during TPC2_{HOTDOWN} compared to

HPC2_{HOTDOWN}. D decreased ($p = 0.01$) during TPC2_{HOTDOWN} compared to TPC1_{TEMPFLAT}. Data presented as median \pm interquartile range.

Figure 8. Relationships between Hsp72 and Hsp90 α mRNA responses in the Vastus Lateralis and Leukocytes immediately before, immediately post, 3 hrs post TPC1_{TEMPFLAT} and HPC1_{HOTDOWN} (all $p < 0.001$).

Figure 9. Individual responses reflecting the change in mRNA (A = Hsp72 mRNA in Vastus Lateralis, B = Hsp90 α mRNA in Vastus Lateralis, C = Hsp72 mRNA in Leukocytes, D = Hsp90 α mRNA in Leukocytes) from baseline to immediately post TPC1_{TEMPFLAT} and TPC2_{HOTDOWN} (left), and HPC1_{HOTDOWN} and HPC2_{HOTDOWN} (right).

In review



All experimental trials consisted of 30 min running at the lactate threshold.
 This corresponded to ~ 70 % VO₂max in the temperate preconditioning group and ~ 66 % VO₂max in the hot preconditioning group.

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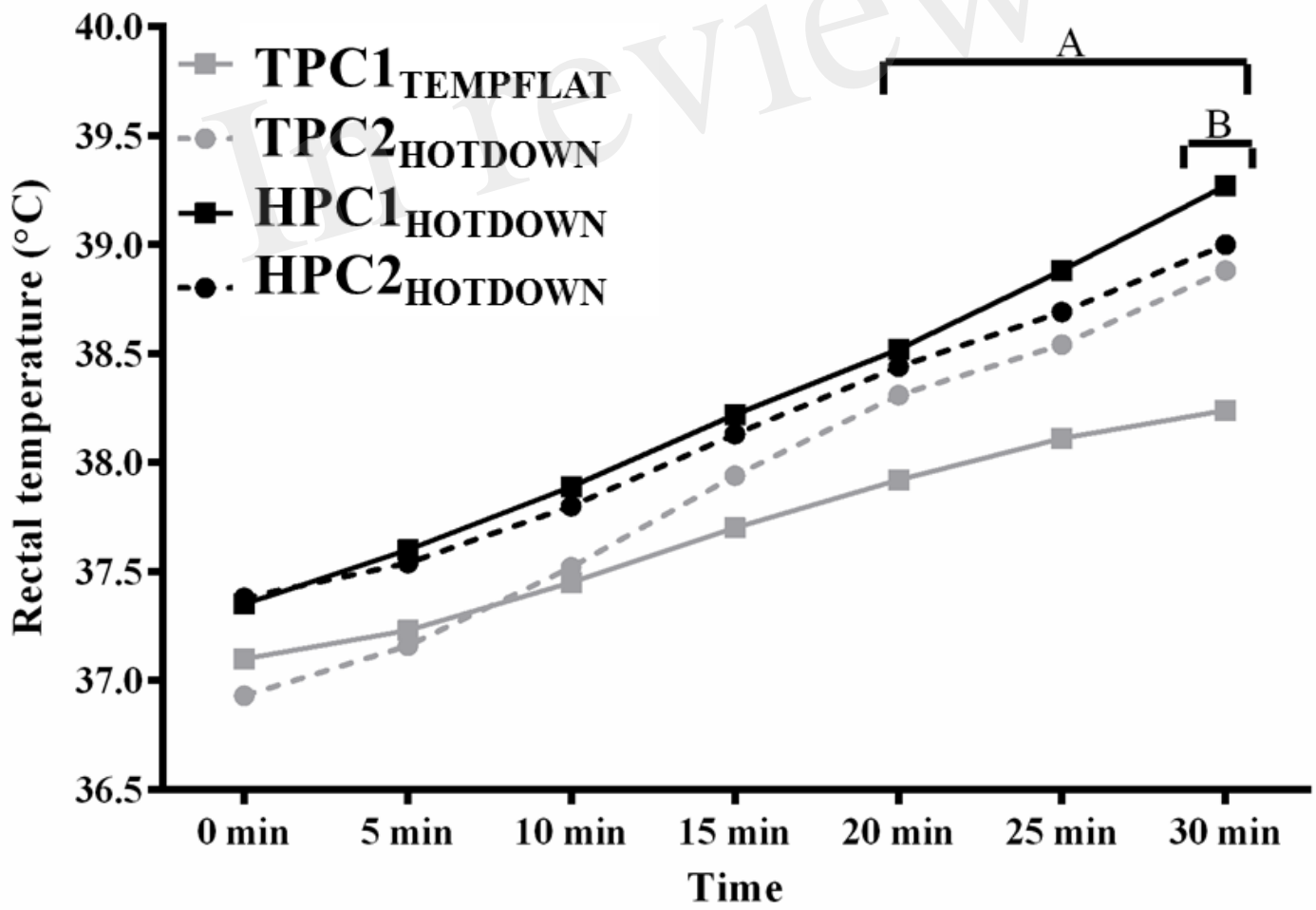


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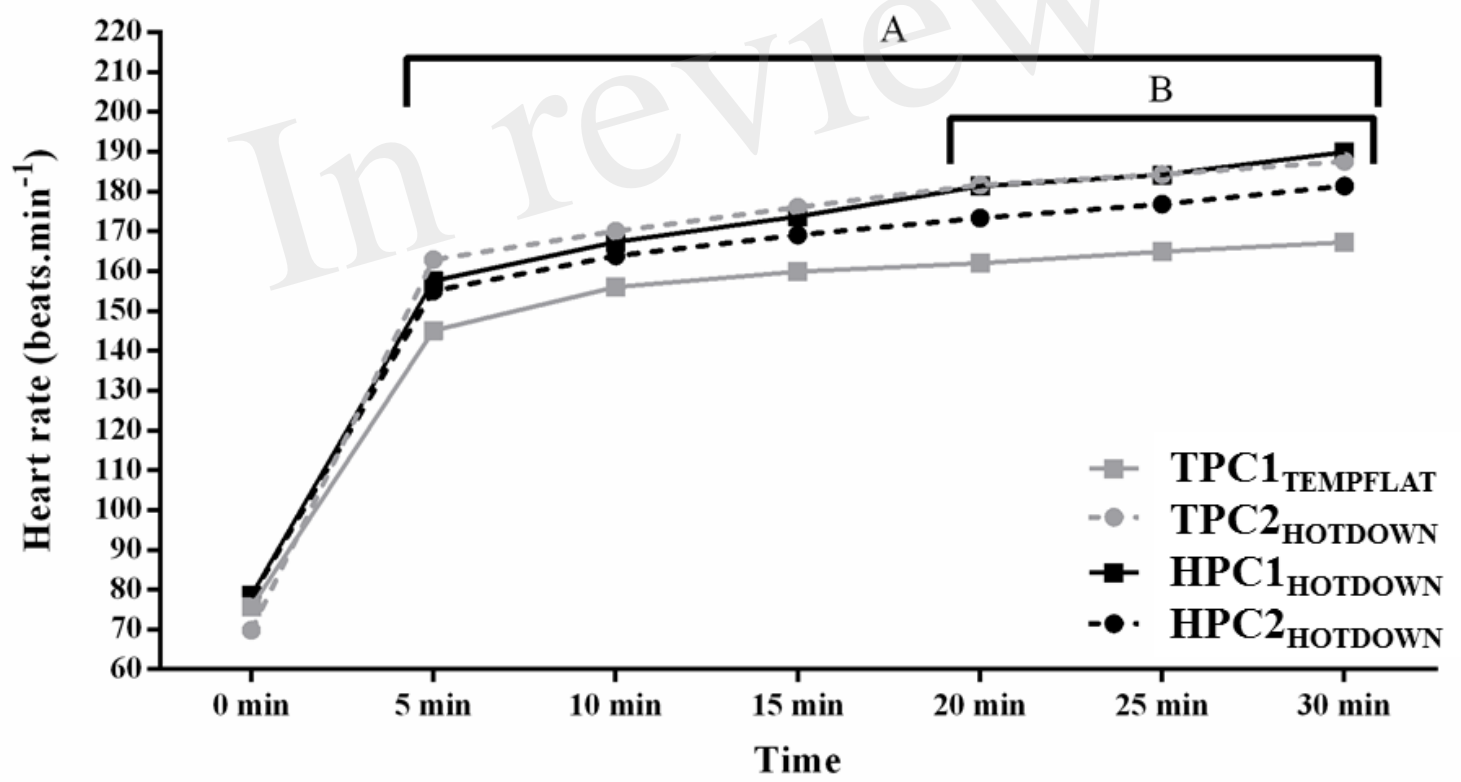


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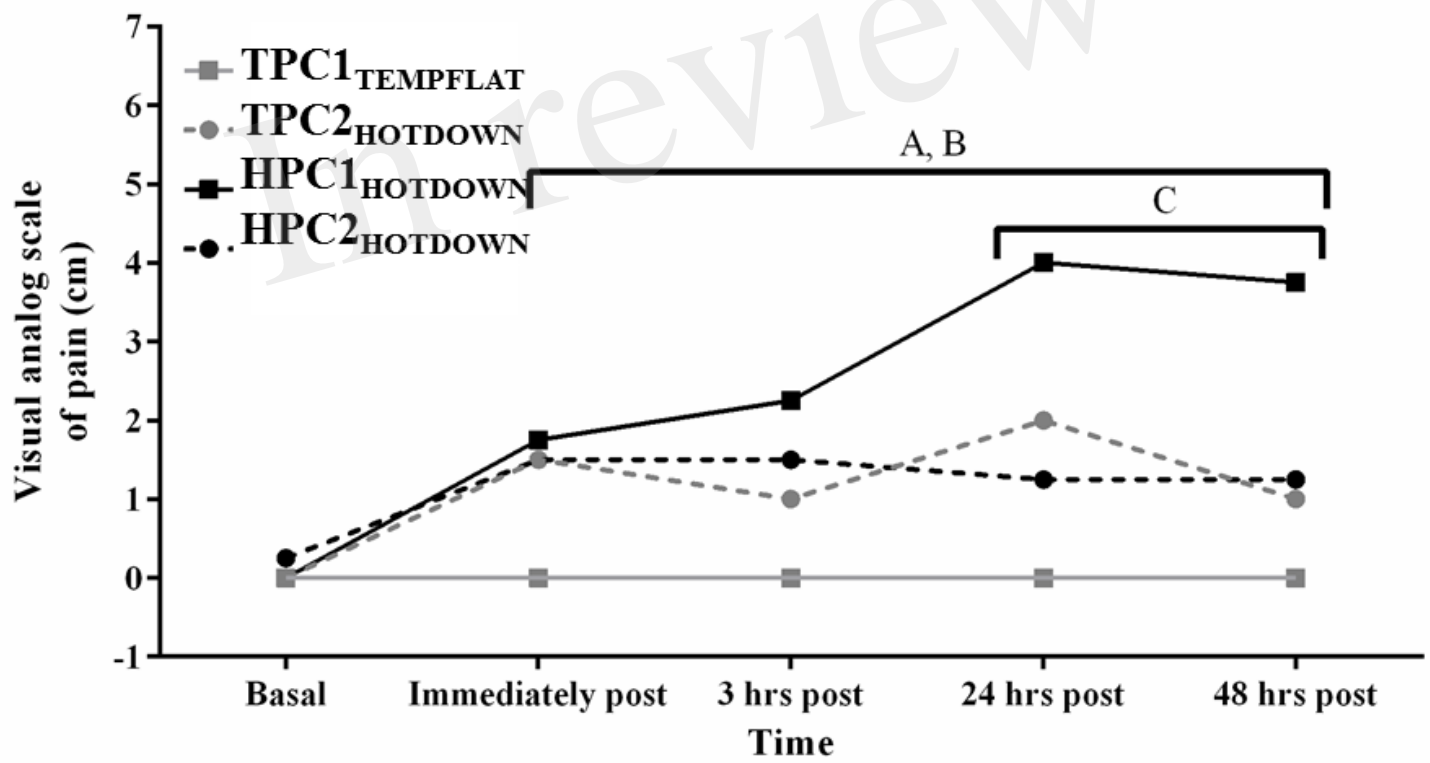


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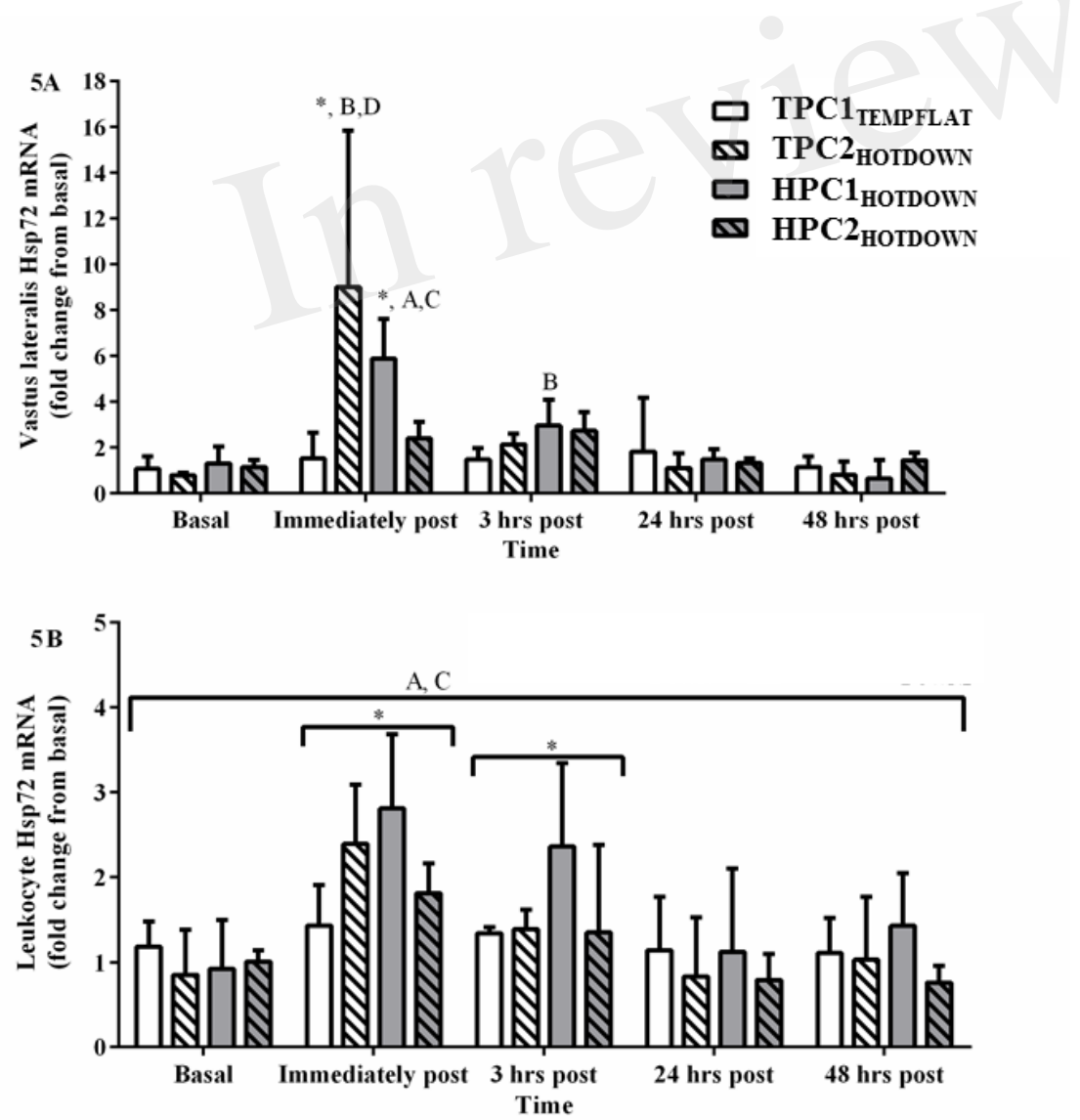


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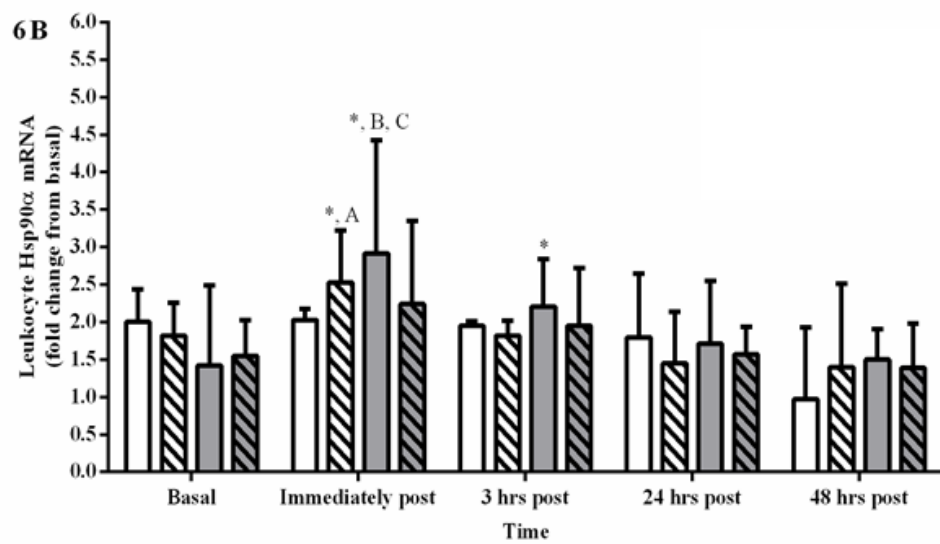
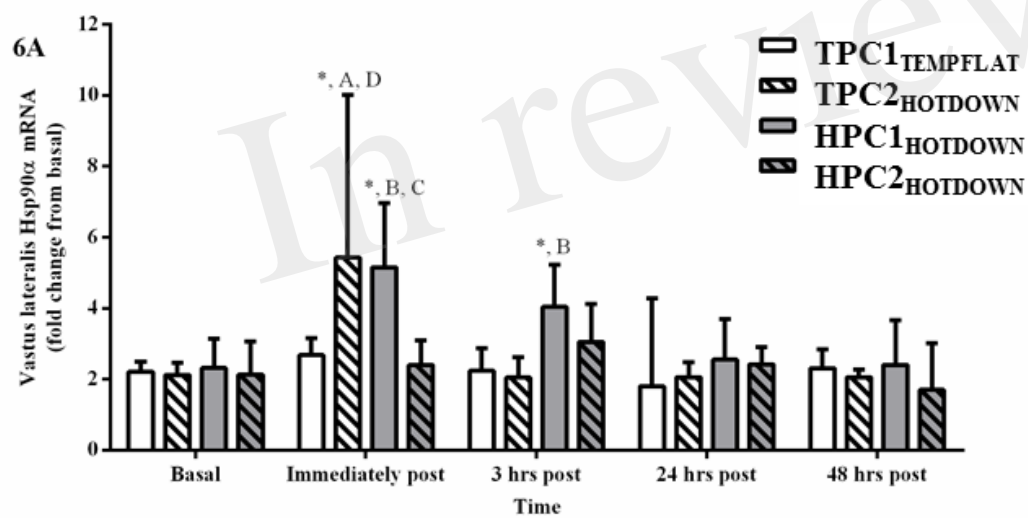


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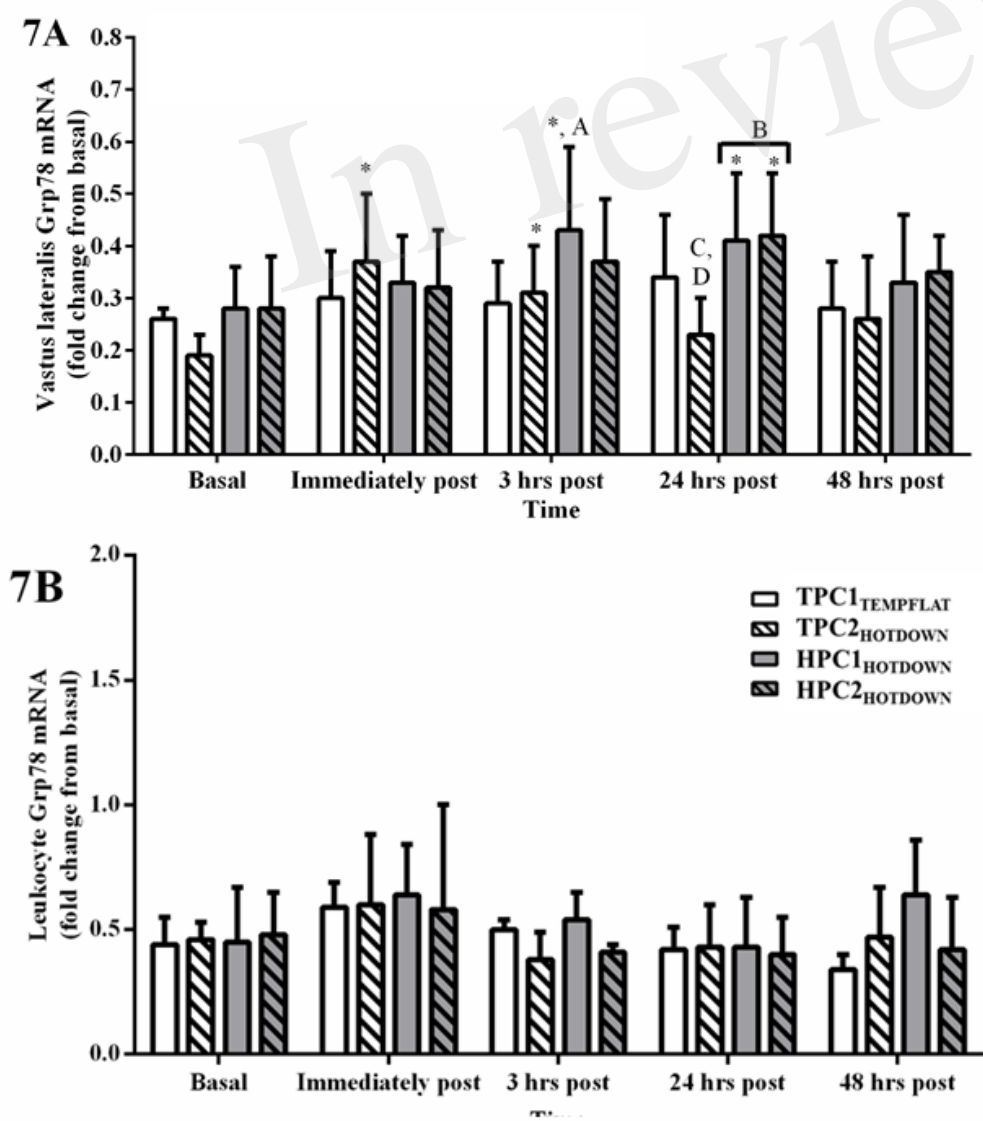


Figure 8.TIF

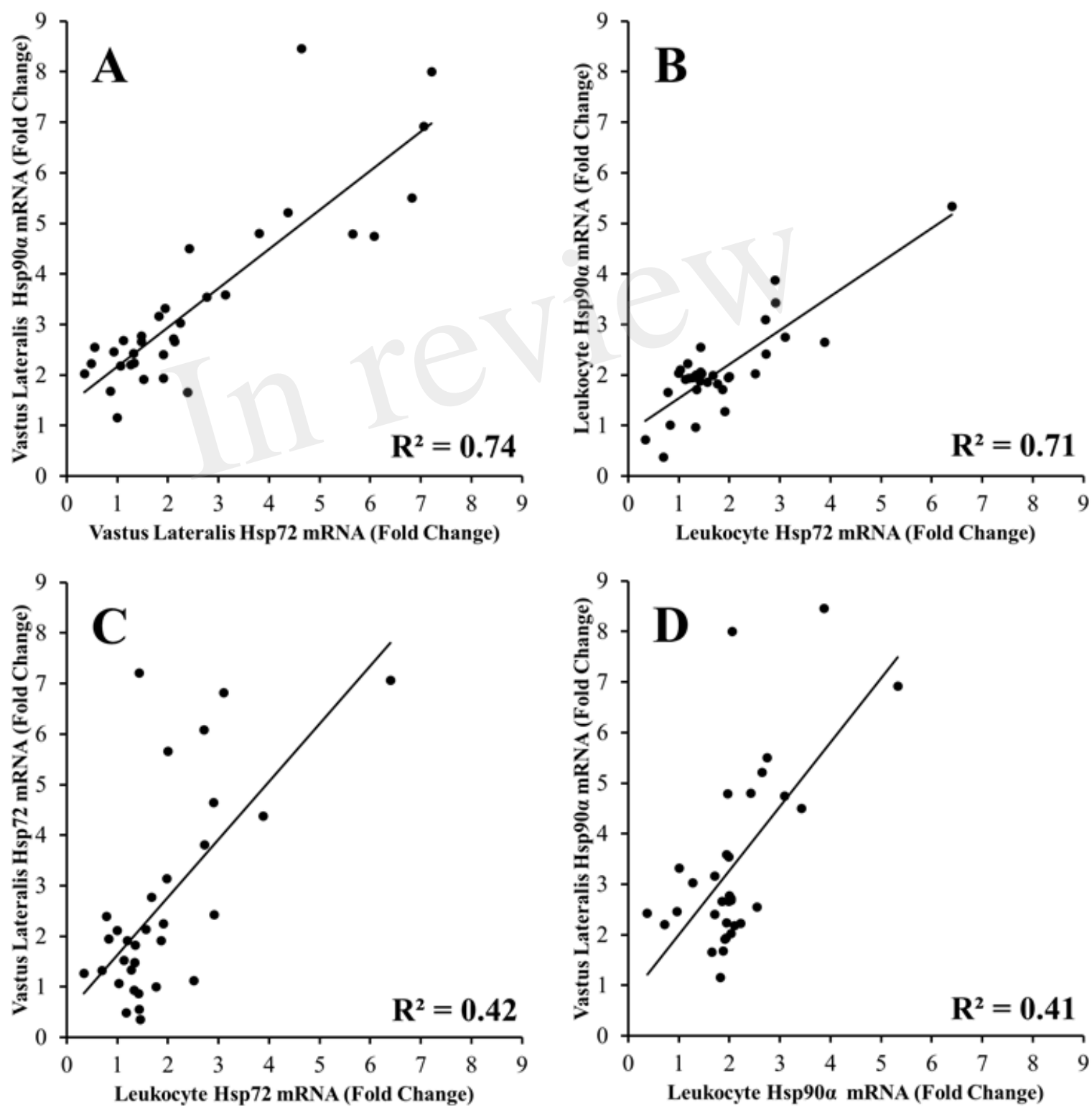


Figure 9.TIF

